METHODS

Mice

All experiments were conducted in accordance with institutional guidelines and approved protocols at UCSF and CSHL. Mice were housed on a 12:12 hour light cycle (lights on 6:00; lights off 180:00) and had ad libitum access to standard chow (LabDiet #5058) or high-fat diet (Research Diets #D12492). *Mc4r*^{loxTB} mice and the *Ai14*$^{fl/fl}$ reporter mice were purchased from Jackson Laboratories and maintained on a C57BL/6J background. *Mc4r*-t2a-*Cre* mice[36] were a generous gift from B. Lowell (BIDMC) and were maintained on a C57BL/6J background. *Esr1*$^{fl/fl}$ were maintained on a mixed background, and *Sf1-Cre*[37] mice were maintained on a C57BL/6N in the lab as previously described [3,4]. Wild type mice used for CRISPRa studies were on a pure C57BL/6J background. For *Mc4r* rescue experiments, the *Sf1-Cre* was contributed through female mice. CUT&RUN experiments were performed on adult male (8-12 weeks of age) gonadectomized C57Bl6/J wild type mice obtained from Jackson Laboratory. Three weeks post-gonadectomy, animals were injected subcutaneously with either corn oil (vehicle) or 5 µg of estradiol benzoate and sacrificed after 4 hours. For each biological replicate, brain dissections were pooled from 5 animals.

Stereotaxic Injections

AAV2-Cre-GFP and AAV2-GFP were purchased from the UNC Vector Core (Chapel Hill, NC). AAV2-hM3Dq-mCherry and AAV2-hM4Di-mCherry were gifts from Bryan Roth and viral preparations were purchased from Addgene (viral prep # 44361-AAV2; http://n2t.net/addgene:44361; RRID:Addgene_44361 and viral prep # 44362-AAV2; http://n2t.net/addgene:44362; RRID:Addgene_44362; Addgene, Watertown, MA)[38]. For axonal tracing, AAV2-CAGs-FLEX-membrane-YFP-WPRE.hGH was re-engineered to fuse YFP with a C-terminal farnesylation tag to enhance the membrane labeling[39]. AAV2 was prepared using a standard polyethylene glycol gradient followed by cesium chloride density gradient centrifugation protocol[40] to reach a titer of (1x10E13 GC/ML). AAVdj-dCas9-VP64 and AAVdj-Prm-Mc4r-sgRNA, were generated by the Stanford Gene Vector and Virus Core and details of vector constructs are as previously described [27],. Adult mice were secured in a Model 1900 stereotaxic frame (David Kopff Instruments, Tujunga, CA), and 250-600 nL of virus was injected bilaterally at the following coordinates: For the VMHvl - A-P: Bregma -1.48 mm, M-L: Bregma +/-0.85 mm, D-V: skull -5.9 mm. For the ARC: A-P: Bregma -1.58 mm, M-L: Bregma +/-0.25 mm, D-V: skull -5.8 mm.

For all surgeries regardless of viral vectors used, mice recovered for at least two weeks prior to any metabolic or behavioral assays. For projection labeling, mice were allowed to express the reporter for 5-8 weeks before tissue collection. At the conclusion of the experiments, mice were euthanized and the brains were collected to confirm proper targeting. Any mice absent of correctly targeted fluorescent protein expression were excluded from subsequent analyses. Water-soluble CNO (HB6149; Hello Bio, Princeton, NJ) was administered by IP injection (0.3 mg/kg in sterile saline) or in the drinking water (0.25 mg/mL). CNO-laden drinking water was replaced every 48 hours. Water-soluble DCZ (HB9126; Hello Bio, Princeton, NJ) was administered in the drinking water (0.1 mg/mL) [41].

Estrous Cycle Staging and EB Treatment

Reproductive stages in female mice were determined by comparing relative amounts of leukocytes, epithelial cells and cornified epithelial cells collected by vaginal lavage. Stage assessments were made daily between ZT3 and ZT5. Brains from estrus or proestrus females were collected between ZT7 and ZT10 and processed for immunofluorescence, ISH, or qPCR.

Adult female mice (>8 weeks old) were OVX. Estradiol benzoate (Cayman Chemical, 10006487) was dissolved in DMSO and diluted in sesame oil (Sigma, S3547). Mice received a subcutaneous injection of either 1ug EB in 150 µL sesame oil or 150 µL of sesame oil with an equivalent amount of DMSO. Control mice received a subcutaneous injection of 150 µL of sesame oil with an equivalent amount of DMSO. To minimize changes in VMH gene expression or signal transduction associated with fear/anxiety, mice were handled daily in a manner that simulated injection for at least 5 days prior to EB/Vehicle treatment and tissue collection. For cFOS analyses, mice were treated with 400µg MT-II (Bachem) by i.p. injection, and brains were collected 1-1.5 hours later.

RNA-seq and qPCR

Brains from OVX females treated with EB (n = 4) or vehicle (n = 3) were rapidly dissected into ice-cold PBS with 0.1% DEPC. Coronal brain sections (250 µm thick) were cut on a vibratome and transferred to glass slides so that the VMH could be visualized and manually microdissected. Isolated tissue was flash frozen and stored at -80°C. RNA was prepared using the RNeasy Micro kit (Qiagen). Sequencing libraries were constructed using the TRIO RNA-seq Library Preparation kit (TECAN) using 15 ng of input RNA. Equal amounts of each sample library were multiplexed and sequenced (50 bp single-end reads) on a single flow cell lane HiSeq 4000 (Illumiina). Demultiplexed reads were aligned to the mouse genome (mm10) using HISAT2[42] v2.1.0 and counted using HTSeq[43] v0.9.1. Finally, differential gene expression testing was performed using DESeq2[44] v1.14.1.

Isolated RNA, prepared as described above, was converted to cDNA using the SuperScript III reverse transcriptase (Invitrogen). qPCR was performed using a BioRad CFX instrument with Maestro software v4.1.2433.1219. Target genes were amplified using specific primers (*Mc4r* forward: 5-GCCAGGGTACCAACATGAAG-3 and reverse: 5-ATGAAGCACACGCAGTATGG-3; *Nmur2* forward: 5-CCTCCTTCCTCTTCTACATCCT-3 and reverse: 5-AGTCACTTTGTCTGCCTCAA-3; *Esr1* forward: 5-GAACGAGCCCAGCGCCTACG-3 and reverse: 5-TCTCGGCCATTCTGGCGTCG-3; and *Ucp1* forward: 5-CACGGGGACCTACAATGCTT-3 and reverse: 5-TAGGGGTCGTCCCTTTCCAA-3). Ct values were normalized to cyclophilin (*Ppib*, forward primer: 5-TGGAGAGCACCAAGACAGACA-3 and reverse primer: 5-TGCCGGAGTCGACAATGAT-3), and relative expression levels were quantified using the comparative C_T method. Individual values, representing the VMHvl or iBAT from 1 mouse are the average of 2 technical replicates.

CUT&RUN

ERα CUT&RUN was performed on 400,000 nuclei isolated from BNSTp, POA, and MeA tissue via density gradient centrifugation[45]. Briefly, tissue was homogenized 15x with a loose pestle in a glass

homogenizer containing Homogenization Medium (250 mM sucrose, 25 mM KCl, 5 mM MgCl2, 20 mM Tricine-KOH, 1 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, 1X Roche EDTA-free protease inhibitor cocktail, pH 7.8). 0.3% IGEPAL CA-630 was added, and the tissue was further dounced 5x with a tight pestle. After douncing, the homogenate was filtered through a 40 µm strainer and mixed 1:1 with 50% OptiPrep solution (Millipore Sigma) prepared in Dilution Buffer (150 mM KCl, 30 mM MgCl2, 120 mM Tricine-KOH, pH 7.8). The homogenate was underlaid with 5 ml of 30% and 40% OptiPrep solution, respectively, and centrifuged at 10,000xg for 18 min at 4°C in an ultracentrifuge. ~2 ml of nuclei solution were removed from the 30 - 40% OptiPrep interface by direct tube puncture. Following nuclei isolation, 0.4% IGEPAL CA-630 was added to improve binding to concanavalin A magnetic beads (Bangs Laboratories BP531). CUT&RUN was performed on brain nuclei, according to the standard protocol[17]. Nuclei were washed twice in Wash Buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% BSA, 0.5 mM spermidine, 1X PIC) and incubated overnight on a nutator with ERa antibody (Millipore Sigma 06-935), diluted 1:100 in Antibody Buffer (Wash Buffer containing 2 mM EDTA). Nuclei were washed twice in Wash Buffer, and ~700 ng/ml protein A-MNase (pA-MNase) was added. After 1 hr incubation on a nutator at 4ºC, the nuclei were washed twice in Wash Buffer and placed in a metal heat block on ice. pA-MNase digestion was initiated by 2 mM CaCl2. After 90 min, pA-MNase activity was stopped by mixing 1:1 with 2X Stop Buffer (340 mM NaCl, 20 mM EDTA, 4 mM EGTA, 50 µg/ml RNase A, 50 µg/ml glycogen). Digested fragments were released by incubating at 37ºC for 10 min, followed by centrifuging at 16,000xg for 5 min at 4ºC. DNA was purified from the supernatant by phenol-chloroform extraction.

CUT&RUN library preparation

CUT&RUN libraries were prepared using the SMARTer ThruPLEX DNA-seq Kit (Takara Bio), with the following PCR conditions: 72ºC for 3 min, 85ºC for 2 min, 98ºC for 2 min, (98ºC for 20 sec, 67ºC for 20 sec, 72ºC for 30 sec) x 4 cycles, (98ºC for 20 sec, 72ºC for 15 sec) x 10 cycles. Samples were size-selected with AMPure XP beads (1.5X right-sided and 0.5X left-sided) to remove residual adapter dimers and large DNA fragments. Individually barcoded libraries were multiplexed and sequenced with paired-end 75 bp reads on an Illumina NextSeq, using the High Output Kit.

CUT&RUN data processing

Paired-end reads were trimmed with cutadapt[46] v3.2.0 to remove low-quality base-calls (-q 30) and adapters. Trimmed reads were aligned to mm10 using Bowtie2[47] v2.4.2 with the following flags: --dovetail --very-sensitive-local --no-unal --no-mixed --no-discordant --phred33. After alignment, duplicate reads were removed using Picard v2.21.6 (http://broadinstitute.github.io/picard/) MarkDuplicates (REMOVE_DUPLICATES=true). De-duplicated reads were filtered by mapping quality (MAPQ > 40) using samtools[48] v1.11.0 and fragment length (< 120 bp) using deepTools v3.5.0 alignmentSieve[49]. After filtering, peaks were called using MACS2 v2.2.7.1 callpeak[50] with a q-value threshold of 0.01 and min-length set to 25. Individual replicate BAM files were normalized by counts per million (CPM) and converted to bigwig tracks, using deepTools bamCoverage (-bs 1, --normalize using CPM). CPM-normalized bigwig tracks for individual EB and vehicle samples (n=3 per condition) were plotted using Gviz[51].

In Situ Hybridization

For colorimetric ISH, antisense *Mc4r* probes were PCR amplified (forward primer: 5-ACTCTGGGTGTCATAAGCCTGT-3 and reverse primer: 5-TCTGTCCCCCACTTAATACCTG-3) from hypothalamic cDNA libraries, and in vitro transcribed with incorporation of digoxigenin-UTP (Roche) using the T7 or SP6 Riboprobe kit (Promega). 20 µm sections from fixed tissue were labeled and detected as previously described[3]. Fluorescent ISH was performed using RNAScope (ACD, Multiplex Fluorescent V2) according to the manufacturer's protocol using the following probes: *Esr1* (478201), *Mc4r* (319181-C2), and *Rprm* (466071).

Immunofluorescent Staining and Histology: Fixed CNS tissue was cryosectioned (20 µm) and stained overnight with primary antibodies against: ERα (EMD Millipore, #C1355 polyclonal rabbit, 1:750 dilution or Abcam, #93021 monoclonal mouse, 1:100 dilution), phospho-Serine 244/247 RPS6 (Invitrogen, #44-923G polyclonal rabbit, 1:500 dilution), cFOS (Santa Cruz, SC-52 polyclonal rabbit, 1:500 dilution), or red fluorescent protein (Rockland, 600-401-379 polyclonal rabbit, 1:1000 dilution). For detection, sections were labeled with species-appropriate secondary Alexa Fluor-coupled antibodies (Invitrogen, #A11029 and #A11037, 1:1000 dilution for both). Widefield Images were acquired using a Nikon microscope and NIS-Elements v3.22.15. Confocal images were acquired at the UCSF Nikon Imaging Center using a Nikon CSU-22 with EMCCD camera and MicroManager v2.0gamma. Images were processed and quantified using ImageJ FIJI v1.52i and the Cell Counter plugin v2.

Fixed gonadal white adipose tissue was paraffin embedded, sectioned (5 µm), and stained with hematoxylin and eosin (H&E) by the Gladstone Histology and Light Microscopy core. Brightfield images were thresholded to define adipocyte borders, and the adipocyte area was quantified using ImageJ FIJI.

Micro-Computed Tomography

Following perfusion fixation, femurs from CRISPRa^{Mc4r} and Control female mice were isolated. Volumetric bone density and bone volume were measured by µCT as previously described[4].

Metabolic and Activity Monitoring

Indirect calorimetry and food intake were measured in CLAMS chambers (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments) and analyzed using CLAX v2.2.15. Any spilled food that was not consumed was accounted for at the conclusion of the four day period spent in CLAMS.

Ambulatory activity in mice subjected to chemogenetic or CRISPRa manipulations was recorded via IR cameras and quantified using the ANY-maze behavioral tracking system (Stoelting, v6.33). Prior to any measurements, mice acclimated to single-housing in the ANY-maze chambers for at least three days. For CRISPRa studies, activity tracking was continuously monitored for at least five 24hr periods.

Interscapular skin temperatures were measured using a FLIR-E4 handheld infrared camera (FLIR Systems, Inc. Wilsonville, Oregon) and the FLIR Tools analysis software v5.13.18031.2002 as previously described. Female mice were lightly anesthetized in groups of four or five in an anesthesia induction chamber and images were captured at baseline, 30 minutes, and 60 minutes post saline, CNO (0.3 mg/kg) or CL-316,243 (3 mg/kg) intraperitoneal injection.

Blood glucose and lipid assays were performed following a 6 hour fast (starting ~ZT2) during which mice were housed in clean cages with ad libitum water access. For glucose and insulin tolerance tests, fasted mice were injected with glucose (I.P., 1 g/kg) or inulin (I.P., 1 U/kg), respectively. Tail-blood samples were collected at baseline and every 15 minutes after glucose/insulin injection. Blood glucose levels were quantified using a hand-held glucometer (Roche, Accu-Check Compact). For triglyceride and cholesterol measurements, plasma was isolated from tail-blood and measured (3 µL in technical duplicates) using the Cholesterol Quantitation Kit (Sigma, MAK043) or the Triglyceride Quantification Colorimetric/Fluorometric Kit (Sigma, MAK266).

Statistics

Statistical tests, excluding RNA-Seq analyses, were performed using Prism 8 (Graphpad). A description of the test and results are provided in **Extended data Table 2**. Multiple comparisons correction for 1-way, 2-way, and repeated measures (RM) ANOVA were performed using the Holm-Šidák post hoc test. Unless otherwise noted, data are presented as mean ± SEM or box plots in which whiskers represent minimum and maximum values, edges of the box are 25th and 75th percentiles, and center line indicates mean. Sample sizes were based on previous work from our lab; however, no specific statistical calculation was performed to determine sample size. For chemogenetic manipulation, mice were drawn at random from a pool of littermate mice containing a roughly equal mix of Cre+ and Cre- genotypes; both mice were injected with the same Cre-dependent AAV construct; partitioning into control and experimental groups was therefore determined by genotype. For AAV-Cre, AAV-mYFP, and CRISPRa injections mice of the identical genotypes were drawn at random from littermate pools to receive functional or control virus injections. Physical activity and food intake parameters were objectively measured using the ANY-maze and/or CLAMS automated systems. Measurements of BAT temperature and mRNA expression as well as cortical bone parameters were made by experimenters blinded to the type of AAV received/genotype of the mice under study. The large differences in body weights among Mc4r control, null, and rescue mice precluded blinding against the genotype of these mice.

Data Availability

RNA-seq and CUT&RUN datasets are available through the NCBI's Gene Expression Omnibus[52] and are identified under the GEO Series accession numbers GSE181204 and GSE141434, respectively. Other supporting data available upon request.

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AUTHOR CONTRIBUTIONS

W.C.K. designed experiments, analyzed data, and wrote the manuscript. R.R. performed thermal and glucose homeostasis analyses in mice. B.G. optimized, performed, and analyzed the CUT&RUN method for ERα binding in neurons. N.M. provided CRISPRa viral vectors and expert advice. A.R. performed histology and quantification of expression data. A.M.P-R. aided with chemogenetic data acquisition and analyses. C.B.H. analyzed bone and plasma lipid data. S.M.C. designed experiments, provided animal models, and analyzed data. K.T. and X.D. provided the AAV-DIO-mYFP vector. N.A. provided key unpublished reagents related to CRISPRa constructs and helped guide studies. J.T. optimized CUT&RUN method for ERα binding in neurons, performed analyses, and wrote the manuscript. H.A.I designed experiments, analyzed data, and wrote the manuscript.

Competing Interests

The authors declare no competing interests.

Supplementary Information

Supplementary Video: CNO administered in the drinking water stimulates VMHvl^{MC4R} neurons to increase physical activity.

Extended Data Figure 1: VMHvl^{ERαKO} does not affect energy intake or physical activity during the light phase but does decrease iBAT thermogenic gene expression.

Extended Data Figure 2: Induction of pS6 and *Mc4r* in the VMHvl depends on estrogen signaling through ERα.

Extended Data Figure 3: ERα binding sites in EB-sensitive target genes contain conserved ERE consensus sequences.

Extended Data Figure 4: Limited induction of *Mc4r* expression in the MeA by ERα signaling, and additional neuroanatomical targets of VMHvl^{MC4R} neurons.

Extended Data Figure 5: VMHvl^{MC4R} neurons function specifically to drive physical activity.

Extended Data Figure 6: Increased physical activity and improvement in metabolic health markers in OVX VMHvl$^{MC4R::hM3Dq}$ females in response to acute and chronic CNO.

Extended Data Figure 7: Estradiol-dependent melanocortin signaling in the VMHvl and additional metabolic and expression data for conditional *Mc4r* rescue mice.

Extended Data Figure 8: Expression and physical activity levels in male and female CRISPRa^{Mc4r} mice.

Extended Data Table 1: Brain region abbreviations

Extended Data Table 2: Statistical tests and results for main Figures 1-4

Extended Data 1: VMHvl^{ERαKO} does not affect energy intake or physical activity during the light phase but does decrease iBAT thermogenic gene expression. a, Representative image of a successful hit confirmed post-mortem by loss of ERα expression (red) in the VMHvl (white arrow) with corresponding expression of GFP driven by the AAV2-Cre viral vector. **b,** X-ambulatory and food intake parameters obtained in light period for VMHvl^{ErαKO}, and ARC^{ERαKO} female cohorts compared to controls (n=7/6). **c,** Equivalent fat mass and **d,** oxygen consumption rates in control, VMHvl^{ERαKO}, and ARC^{ERαKO} female mice measured by EchoMRI and indirect calorimetry in CLAMS, respectively (see main figure for number of mice/group). **e**, Quantification of BAT thermogenic gene expression levels by qPCR in VMHvl^{ERαKO} (n=6), ARC^{ERαKO} (n=7), and control (n=6/3) female mice (VMHvl^{ERαKO} *Ucp1*: unpaired 2-tailed *t* test, $t_{(9)}$=2.599, *P*=0.0288).

Extended Data Figure 2: Induction of pS6 and *Mc4r* in the VMHvl depends on estrogen signaling through ERα. a, IF of ERα and pS6$^{244/47}$ in the VMHvl (left panels) and ARC (right panels) of *Esr1$^{fl/fl}$* and OVX female mice 4 hrs post EB treatment. **b**, IF of ERα (red) and pS6$^{244/47}$ (green) in the VMHvl in conditional knockout (*Esr1$^{Nkx2\text{-}1Cre}$*) OVX female mice following 4 hrs post EB treatment. **c,** IF of ERα (red) and pS6$^{244/47}$ (green) in the VMHvl of intact adult male mice following 4 hrs post vehicle or EB treatment. Bar graph to the right shows increased number of pS6$^{244/47}$+ neurons in the VMHvl of male mice post EB treatment as done for OVX females (unpaired 2-tailed *t* test, $t_{(6)}$=8.569, *P*=0.0001). **d,** Mammalian Phenotype Ontology terms most significantly enriched and top five significantly enriched Reactome Pathways among DEGs in the VMHvl (vehicle vs. EB). **e**, qPCR analysis of the indicated target genes in VMHvl from estrus females, proestrus females, and males (♂); data points represent values from individual mice (1-way ANOVA *Nmur2*: $F_{(2,15)}$=8.469, *P*=0.0035, post hoc: E vs P *P*=0.0454, P vs ♂ *P*=0.0030, and E vs ♂ P=0.1438; *Esr1*: $F_{(2,11)}$=10.18, *P*=0.0031, post hoc: E vs P *P*=0.1650, P vs ♂ *P*=0.0374, and E vs ♂ *P*=0.0033). Holm-Šidák multiple comparisons. **f**, *Mc4r* expression levels in VMHvl from OVX EB treated (n=6) females normalized to vehicle treatment (n=3) (unpaired 2-tailed *t* test $t_{(6)}$=6.519, *P*=0.0006). **g,** ISH showing *Mc4r* expression (red arrows) in the VMHvl of an estrus female, a proestrus female, and an intact male. **h,** Representative ISH (*Mc4r*, red arrows) and immunofluorescent (pS6, yellow arrows) staining in the VMHvl (dashed circle) from OVX female mice treated with vehicle for 4 hrs, EB for 2 hrs, or EB for 4 hrs. **i,** Full size images showing bilateral expression of *Mc4r* and *Rprm* in intact females staged for estrus and proestrus. *Rprm* expression is unchanged in both estrous stages. Data presented as box plots (see Fig. 1c legend for description). Micrographs are representative of data from 5 mice.

Extended Data Figure 3: ERα binding sites in EB-sensitive target genes contain conserved ERE consensus sequences. CUT&RUN CPM-normalized coverage track showing EB-specific ERα binding

sites containing EREs (pink boxes) within the **a,** *Greb1* locus (3/3 replicates) and **b,** *Pgr* locus (3/3 replicates), and in the **c,** *Mc4r* promoter (1 of 3 replicates) in 400,000 sub-cortical brain nuclei collected from vehicle and EB (5 µg) treated gonadectomized mice. Below each track the location and sequence conservation of full (*a,b*) ERE and half (*c*) SP1/ERE consensus sites in target gene loci indicated by pink and green boxes. **d, e,** Location and sequence conservation of ERE consensus sites within *Mc4r* and *Nmur2* loci corresponding to ERα binding sites presented in **Fig 1g**. For all panels the genomic intervals containing ERE/SP1 sites are located within the ERα binding sites identified by CUT&RUN.

Extended Data Figure 4: Limited induction of *Mc4r* expression in the MeA by ERα signaling, and additional neuroanatomical targets of VMHvl^{MC4R} neurons. a, Representative coronal brain images of Ai14^{Mc4r} female mice stained for ERα (green) and Ai14 (magenta) in the MeA used for quantification shown in **Fig 2a**. **b,** Additional ISH comparing *Mc4r* induction in the VMHvl and MEA in estrus and proestrus females. **c**, Representative mYFP reporter expression in additional neuroanatomical regions (scale bars=200µm). **d**, Heatmap from **Fig. 2d** rearranged to compare VMHvl^{MC4R} and VMHvl^{ERα} projection intensity in target regions along the anterior-posterior axis. **e**, VMHvl^{MC4R} projections to the PAG preferentially target the PAGdl/l and PAGdm (scale bars=200µm).

Extended Data Figure 5: VMHvl^{MC4R} neurons function specifically to drive physical activity. a, Distance traveled over time in female and male mice following a single injection of CNO (RM 2-way ANOVA interaction effect female: $F_{(39,312)}$=11.96, P<0.0001; male: $F_{(39,312)}$=6.898, P<0.0001). **b**, Total time spent immobile in intact female and male VMHvl^{Cre-} controls (n=5/4) and VMHvl$^{MC4R::hM3Dq}$ mice (n=5/5) (RM 2-way ANOVA female interaction effect $F_{(1,8)}$=33.89, P=0.0004, post hoc P<0.0001 and male interaction effect $F_{(1,8)}$=96.79, P=0.0005, post hoc P<0.0001). **c**, Thermal imaging of BAT surface temperatures for VMHvl^{Cre-} (left mouse) and VMHvl$^{MC4R:: hM3Dq}$ (right mouse) females treated with CL. **d,** No differences were observed in *Ucp1* mRNA in the BAT from VMHvl^{Cre-} and VMHvl$^{MC4R::hM3Dq}$ mice collected 1.5 hrs after a single CNO injection. **e**, Body weights for female VMHvl^{Cre-} (n=5) and VMHvl$^{MC4R::hM3Dq}$ (n=6, baseline measurement includes 1 mouse with mistargeted injection) mice prior to glucose tolerance test (GTT). **f,** GTT glucose levels for intact female cohorts treated with saline or CNO. **g**, Body weight normalized food consumption in females (n=5/5) following Sal/CNO injection during light dark period (ZT12-17) (RM 2-way ANOVA Dark period interaction effect $F_{(1,8)}$=3.502, P=0.0982, post hoc P=0.0489). **h,** Sustained physical activity increase across light/dark periods in VMHvl$^{MC4R::hM3Dq}$ (n=5) females administered CNO-H2O as compared to VMHvl^{Cre-} females (n=5) or during exposure to plain drinking water (H_2O). **i,** Cumulative distance traveled and **j,** number of rearing events light/dark periods following administration of CNO or water during the light stage (*i* RM 2-way ANOVA $F_{(1,8)}$=15.8,

P=0.0041, post hoc P=0.0006 and *j*, RM 2-way ANOVA $F_{(1,8)}$=15.8, P=0.0041, post hoc P=0.0006). **k**, Starting body weights and weight change during continuous administration of CNO-H_2O for intact females. **l,** ERα and mCherry expression in the VMHvl following targeted injection of Cre-dependent AAV-hM4Di-mCherry into a female *Mc4r-t2a-Cre* mouse. **m,** Number of rearing episodes during the dark period in VMHvl$^{MC4R::hM4Di}$ (n=8) and VMHvl^{Cre-} (n=4) intact female mice following administration of plain or DCZ-laden drinking water. Data are mean ± SEM or box plots (described in Fig. 1c legend). Holm-Šidák multiple comparisons test as appropriate.

Extended Data Figure 6: Increased physical activity and improvement in metabolic health markers in OVX VMHvl$^{MC4R::hM3Dq}$ females in response to acute and chronic CNO. a, Distance traveled over time in OVX female VMHvl^{Cre-} (n=5) and VMHvl$^{MC4R::hM3Dq}$ (n=5) mice during administration of plain H_2O or CNO-H_2O (RM 2-way ANOVA interaction effect $F_{(11,88)}$=5.265, P<0.0001). **b,** Total dark period rearing events in intact and OVX females administered plain H_2O or CNO-H_2O drinking water (RM 2-way ANOVA interaction effect $F_{(1,8)}$=60.31, P<0.0001 post hoc P<0.0001). **c**, Body weights (RM 2-way ANOVA time effect $F_{(2,24)}$=49.51, P<0.0001; genotype effect $F_{(2,24)}$=33.50, P<0.0001) and fasting glucose levels (RM 2-way ANOVA time effect $F_{(2,26)}$=6.456, P=0.0053; genotype effect $F_{(1,26)}$=10.11, P=0.0038) in female mice after OVX and subsequent HFD feeding. **d,** Blood glucose (left) and AUC (right) during ITT in chow-fed OVX females following 6-hour fast and saline/CNO treatment. **e,** Blood glucose (left, RM 2-way ANOVA interaction effect $F_{(1,8)}$=7.791, P=0.0235, post hoc: VMHvl$^{MC4R::hM3Dq}$ saline vs CNO, T15 P=0.0009 and T60 P=0.0318) and AUC (right, RM ANOVA with mixed-effects model, note: one *Cre*$^{+}$ female with missed injection was included in saline but not CNO treated groups, interaction effect $F_{(1,8)}$=7.791, P=0.0235, post hoc: VMHvl$^{MC4R::hM3Dq}$ saline vs CNO P=0.0007) values during 90 min ITT test on HFD-fed OVX females performed 6 hours post fasting and post injection with saline or CNO. **f**, Blood glucose levels following a 6 hr fast in OVX females maintained on Chow/HFD following a single saline or CNO injection (RM ANOVA with mixed-effects model, note: one *Cre*$^{+}$ female with missed injection was included in saline but not CNO treated groups, Chow: treatment effect $F_{(1,17)}$=5.038, P=0.0384, post hoc P=0.0179; and HFD: interaction effect $F_{(1,17)}$=20.47, P=0.0019, post hoc P=0.0073). **g**, Percent change in body weight in HFD-fed OVX females (n=5/5) continuously administered CNO-laden drinking water (RM 2-way ANOVA interaction effect $F_{(7,64)}$=4.583, P=0.0003. **h,** Fasting blood glucose levels in OVX/HFD mice before and after 8 days of chronic CNO (RM ANOVA with mixed-effects model, note: one *Cre*$^{+}$ female with missed injection was included in saline but not CNO treated groups, interaction effect $F_{(1,17)}$=5.180, P=0.0361 post hoc: VMHvl$^{MC4R::hM3Dq}$ Pre vs Post P=0.0156). **i**, Plasma cholesterol levels before (Pre) and after (Post) 8 days of continuous CNO-H_2O exposure (RM ANOVA with mixed-effects model, note: one *Cre*$^{+}$ female with missed injection was included in saline but not CNO treated

groups, interaction effect $F_{(1,8)}$=5.502, P=0.0470, post hoc P=0.0203). **j,** Average daily food intake during 8 days of continuous CNO-H_2O exposure (points represent separate daily measurements of average consumption per mouse). Data are mean ± SEM or box plots (described in Fig. 1c legend). **a, b, c, d, e, f, g, h,** Holm-Šidák multiple comparisons test as appropriate.

Extended Data Figure 7: Additional metabolic and expression data for conditional *Mc4r* rescue mice. **a**, cFos expression (arrows) in the VMHvl and PVH of female mice treated with EB ± MT-II. **b**, cFos+ cells in EB+MT-II (n=5) compared to Veh+MT-II (n=3, **P=0.0037) and EB+Veh (n=4, **P=0.0046) treated females (1-way ANOVA $F_{(2,9)}$=14.00, P=0.0017). **c**, *Mc4r* and *Sf1* expression patterns from Genotype-Tissue Expression Project intersect specifically in the hypothalamus (blue arrows) and not in peripheral tissues (red arrows). Transcripts/million (TPM) expression presented as box plots with center line at median, box edges are 25th and 75th percentiles, and whiskers are 1.5x interquartile range. **d**, Equivalent body weights within cohorts of female and male $Mc4r^{+/+}$, $Mc4r^{loxTB}$, and $Mc4r^{Sf1\text{-}Cre}$ mice at weaning. **e**, % lean (1-way ANOVA $F_{(2,31)}$=101.4, P<0.0001, post hoc: $Mc4r^{+/+}$ vs $Mc4r^{loxTB}$ P<0.0001, $Mc4r^{+/+}$ vs $Mc4r^{Sf1\text{-}Cre}$ P<0.0001, and $Mc4r^{Sf1\text{-}Cre}$ vs $Mc4r^{loxTB}$ P=0.0720) and % fat (1-way ANOVA $F_{(2,31)}$=104.2, P<0.0001, post hoc: $Mc4r^{+/+}$ vs $Mc4r^{loxTB}$ P<0.0001, $Mc4r^{+/+}$ vs $Mc4r^{Sf1\text{-}Cre}$ P<0.0001, and $Mc4r^{Sf1\text{-}Cre}$ vs $Mc4r^{loxTB}$ P=0.0769) body composition analysis (EchoMRI) in adult females of each genotype. **f,** Oxygen consumption (VO2) as a function of body weight in adult female mice. **g,** Body weights in 13-week-old $Mc4r^{+/+}$, $Mc4r^{loxTB}$, and $Mc4r^{Sf1\text{-}Cre}$ females (1-way ANOVA $F_{(2,32)}$=226.6, post hoc: $Mc4r^{+/+}$ vs $Mc4r^{loxTB}$ P<0.0001, $Mc4r^{+/+}$ vs $Mc4r^{Sf1\text{-}Cre}$ P<0.0001, and $Mc4r^{Sf1\text{-}Cre}$ vs $Mc4r^{loxTB}$ P=0.0029). Data presented as mean ± SEM or scatterplots of values from individual mice. Holm-Šidák multiple comparisons test as appropriate.

Extended Data Figure 8: Expression and physical activity levels in male and female CRISPRa^{Mc4r} mice. a, mCherry expression in CRISPRa^{Mc4r} female hypothalamus. **b**, Fluorescent ISH images from CRISPRa^{Mc4r} female (left) and male (right) showing *Esr1* and *Mc4r* expression. Images are reproduced from **Fig. 4g** to show limited induction of *Mc4r* outside of the VMHvl target region. **c**, Dark phase (ZT12-24) physical activity levels (distance/12 hours) as a function of *Mc4r* or *mCherry* mRNA expression in microdissected VMHvl from control and CRISPRa^{Mc4r} female mice. **d,** Home-cage activity in CRISPRa^{Mc4r} (n=4) and control (n=3) male mice. **e**, Time spent immobile during the 12 hour dark phase in control and CRISPRa^{Mc4r} female (unpaired 2-tailed t test, $t_{(9)}$=2.015, P=0.0747) and male CRISPRa^{Mc4r} mice (see main figure for number of mice/group) (unpaired 2-tailed t test, $t_{(5)}$=3.245, P=0.0228) mice. **f,** CRISPRa^{Mc4r} (n=6) and control (n=7) female body weights during ad lib feeding. **g,** Normalized daily food intake in CRISPRa^{Mc4r} (n=6) and control (n=7) females (unpaired 2-tailed t test, $t_{(11)}$=2.409, *P=0.0347). **h,** BAT

surface temperatures in female control (n=4) and CRISPRa^{Mc4r} (n=5) mice, repeated measurements at 30- and 60-min post-anesthesia. **i,** Cortical bone thickness for female cohorts (unpaired 2-tailed *t* Test, $t_{(6)}$=2.957, *P*=0.0254). **j**, Body weights in control (n=4) and CRISPRa^{Mc4r} (n=6) females at wk 9 and at wk 17 after eight weeks of OVX. **k**, Distance traveled over 24 hours in OVX control and OVX CRISPRa^{MC4R} compared to intact females (blue). **l,** total dark phase distance in intact (n=5), OVX control (n=7), and OVX CRISPRa^{Mc4r} (n=8) females. Data are presented as scatterplots of values from individual mice, mean ± SEM, or as box plots (described in Fig. 1c legend).